

INFECTION RATES, PARASITEMIA LEVELS, AND GENETIC DIVERSITY OF
HEMATOZOA IN NEW WORLD WATERFOWL

By

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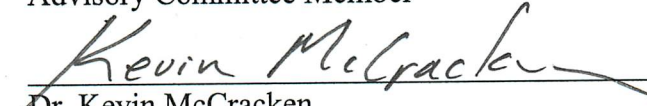
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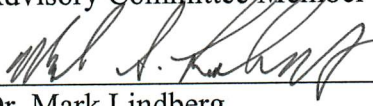
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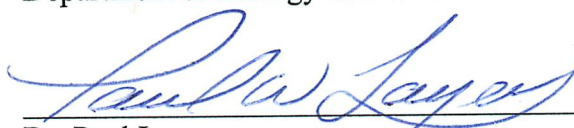


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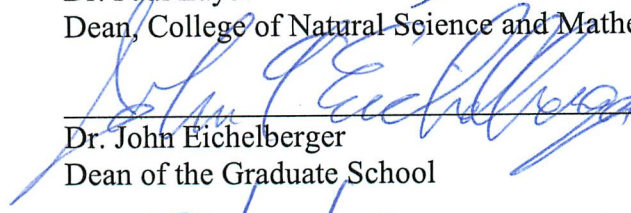


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INFECTION RATES, PARASITEMIA LEVELS, AND GENETIC DIVERSITY OF
HEMATOZOA IN NEW WORLD WATERFOWL

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By
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ABSTRACT

Blood parasites can limit the productivity of birds and increase the vulnerability of isolated and naïve populations to extinction. I examined 804 blood samples collected from 11 species of South American waterfowl to assess infection by *Haemoproteus*, *Plasmodium* and/or *Leucocytozoon* parasites. In addition, I strove to develop a new molecular tool to quickly and accurately determine relative parasitemia rates of *Leucocytozoon* parasites in avian blood. I used samples collected from waterfowl in interior Alaska ($n = 105$) to develop and optimize a real-time, quantitative PCR methodology using TaqMan fluorogenic probes.

Molecular screening produced an apparent prevalence rate of 3.1% for hematozoa infections in South American waterfowl samples, and analysis of hematozoa mitochondrial DNA produced 12 distinct hematozoa haplotypes, four of which were identical to hematozoa lineages previously found infecting waterfowl in North America. Phylogenetic analyses of hematozoa DNA revealed close relationships between parasite lineages infecting waterfowl on both continents. Our qPCR assay showed high levels of sensitivity (91%) and specificity (100%) in detecting *Leucocytozoon* DNA from host blood when compared to results from a well-used nested-PCR protocol. Additionally, statistical results of a linear regression supported correlation between relative parasitemia estimates from our qPCR assay and greater numbers of parasites observed on blood smears ($R^2 = 0.67$, $P = 0.003$).

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GENERAL INTRODUCTION

Avian haemosporidians belong to a large and phylogenetically diverse group of protozoan blood parasites that infect amphibians, reptiles, birds, and mammals using blood-sucking dipteran insects as vectors (Valkiūnas, 2005). Although the most well known of these protozoans are the human malaria parasites (i.e. *Plasmodium vivax*, *Plasmodium falciparum*, etc.), haemosporidian infections in avian species have been studied for over a century. To date, there have been over 200 species of haemosporidians described for avian hosts (Valkiūnas, 2005). Representative species from the genera *Haemoproteus*/*Parahaemoproteus*, *Plasmodium*, and *Leucocytozoon*, have been detected on every continent except Antarctica (Beadell et al., 2009), in both domestic and wild birds across a broad range of habitat types (Greiner et al., 1975; White et al., 1978). Studies have shown that the most severe pathological effects of haemosporidian infections occur in domestic bird species (e.g., Anderson et al., 1962; Cellier-Holzem et al., 2010), whereas infections in most wild populations appear to be relatively benign. Mortality in wild birds is typically only observed when parasites undergo range expansion and colonize new habitats and infect novel host species (e.g. Hill et al., 2010; van Riper et al., 1986). Sub-lethal effects on wild birds, such as reduced breeding productivity or decreased mobility, remain largely unstudied.

Hematozoa infections in avian hosts were traditionally studied using microscopic examination of peripheral blood smears, allowing researchers to simultaneously diagnose a sample as negative or positive for hematozoa infection, and quantify the infection level (parasitemia) of that sample. Within the last several decades, powerful new molecular

methods employing polymerase chain reaction (PCR) techniques have drastically increased our knowledge of blood parasite diversity, allowing researchers to compare DNA sequence data obtained from parasites infecting different hosts. High-throughput screening of blood/tissue samples is now possible, with the added advantage of identifying unique lineages of parasite mitochondrial DNA (mtDNA) through the sequencing of PCR products. These methods have a disadvantage, however, in that they provide no measure of the level of parasite infection; they give simply a positive or negative result.

Since haemosporidian infections were first described in avian species in 1884, the majority of work has focused on species belonging to the order Passeriformes. In comparison with the studies involving these perching bird species, research on haemosporidian infection dynamics in waterfowl (Anseriformes) has been much less extensive. Species of *Haemoproteus*/*Parahaemoproteus*, *Plasmodium*, and *Leucocytozoon* are commonly found infecting waterfowl, and species of ducks and geese have often been used as hosts in studies involving experimental infection (Anderson et al., 1962; Fallis et al., 1954). Additionally, many waterfowl species are gregarious and migrate long distances between breeding/wintering locations. Such behavior is thought to increase transmission of blood parasites, as high densities of hosts would cause increased transmission by insect vectors (Matta et al., 2014). Information on haemosporidian infections in wild waterfowl populations could provide insight into whether these migrant species are transferring blood parasites into new regions or into naïve species that have not been previously exposed to specific parasite lineages.

My main objectives for this project were to use molecular methods to screen blood samples collected from South American waterfowl for infection by *Haemoproteus*,

Plasmodium, and *Leucocytozoon* blood parasites (chapter 1) and to develop a novel molecular methodology to determine the relative parasitemia level in avian blood samples through the use of real-time, quantitative PCR (chapter 2).

The studies that have examined haemosporidian parasite infections in North American waterfowl (e.g. Bennett et al., 1981; Greiner et al., 1975; Ramey et al., 2012, 2013, 2014) have found moderate to high levels of prevalence and genetic diversity. Little work has been done, however, on the prevalence of avian hematozoa in waterfowl species in South America. White et al. (1975) conducted a meta-analysis of avian haemosporidians in the Neotropics and based on microscopic screening, found a 2.2% prevalence rate among waterfowl species sampled throughout South America. Based on the lack of information published on blood parasites in South American waterfowl in general and almost a complete lack of molecular data on these haemosporidian infections, my primary goal for the first chapter was to obtain information about the prevalence rates of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* parasites in South American waterfowl samples, and to identify any unique parasite haplotypes present. A second goal was to compare these haplotypes with recently published parasite lineages from North American waterfowl to determine if intercontinental transmission of parasite lineages is occurring.

My genetic comparison of parasite lineages between continents was only possible through the use of molecular diagnostic techniques and phylogenetic analysis. Historically, researchers used light microscopy to diagnose and quantify blood parasite infection. This method remains the standard for determining a host's parasitemia level, through the visual identification and quantification of infected erythrocytes. While this method is effective and well accepted, it can be time consuming and requires a high level of training to guarantee

accuracy. Therefore, the focus for the second chapter of my project was to design a molecular methodology using real-time, quantitative PCR (qPCR) to quickly and accurately determine the level of *Leucocytozoon* spp. infection in avian blood samples. This would provide researchers an additional tool for determining a host's parasitemia level without the need for more time-consuming microscopy, and allow the level of parasitemia to be incorporated as a covariate in models that seek to identify factors driving individual variation in demographic rates.

Several studies have produced qPCR methodologies for the quantification of *Haemoproteus* and *Plasmodium* parasites (e.g., Friedl and Groscurth, 2011; Knowles et al., 2010a). To my knowledge, however, there have been no qPCR protocols developed specifically for *Leucocytozoon* parasites. Such a protocol would be beneficial when research questions are focused on non-lethal effects of *Leucocytozoon* infection on avian species, particularly with large sample sizes that would be very time consuming to screen with microscopic methods.

CHAPTER 1

Prevalence and genetic diversity of hematozoa in South American waterfowl and evidence for intercontinental redistribution of parasites by migratory birds¹

Abstract: To understand the role of migratory birds in the movement and transmission of hematozoa within and between continental regions, we examined 804 blood samples collected from eleven species of South American waterfowl in Peru and Argentina for infection by *Haemoproteus*, *Plasmodium*, and/or *Leucocytozoon* blood parasites. Infections were detected in 25 individuals of six species for an overall apparent prevalence rate of 3.1%. Analysis of hematozoa mitochondrial DNA revealed twelve distinct parasite haplotypes infecting South American waterfowl, four of which were identical to lineages previously observed infecting ducks and swans sampled in North America. Analysis of parasite mitochondrial DNA sequences revealed close phylogenetic relationships between lineages originating from waterfowl samples regardless of continental affiliation. In contrast, more distant phylogenetic relationships were observed between parasite lineages from waterfowl and passerines sampled in South America for *Haemoproteus* and *Leucocytozoon*, suggesting some level of host specificity for parasites of these genera. The occurrence of identical parasite lineages in North America and South America, paired with

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the close phylogenetic relationships of hematozoa infecting waterfowl on both continents, provides evidence for parasite dispersal between these regions through migratory birds.

Keywords: Hematozoa; cytochrome *b*; South America; waterfowl; blood parasites; *Anatidae*

Introduction

Protozoan blood parasite infections have been studied in avian species for more than a century (Valkiūnas, 2005), and representative species of parasites from the genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* have been detected on every continent except Antarctica (Beadell et al., 2006; Valkiūnas, 2005). Studies have shown that these hematozoa infections can have adverse fitness effects on individuals of certain avian species (Anderson et al., 1962; Valkiūnas, 2005; Van Riper et al., 1986). Individuals of host populations that are restricted to islands, or host species that have not previously been exposed to hematozoa have been shown to be vulnerable to pathogenic effects of these parasites. For example, introduction of *Plasmodium* parasites to the Hawaiian Islands contributed to the population declines of native bird species (Van Riper et al., 1986). Additionally, *Leucocytozoon* parasites have been associated with high mortality rates in Yellow-eyed Penguin (*Megadyptes antipodes*) chicks in New Zealand (Hill et al., 2010), and were reported to cause mortality in experimentally infected domestic waterfowl (Anderson et al., 1962). In contrast, wild species of waterfowl infected with *Leucocytozoon* parasites have shown few clinical signs of infection (Shutler et al., 1996), although potential sub-

lethal effects such as reduced clutch size and lower survival rates have not been well studied.

In South America, blood parasites belonging to the genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* have been detected in a broad range of avian families throughout the continent (e.g. Bennett et al., 1991; Durrant et al., 2006; Valkiūnas et al., 2003; White et al., 1978). However, only 2.2% of samples collected from waterfowl (family *Anatidae*) in the Neotropics were identified as positive for hematozoa infection (White et al., 1978). At least ten species of South American waterfowl have been sampled and tested for hematozoa infection. Only *Haemoproteus* and *Plasmodium* parasites were detected and infections were limited to three host species: Brazilian Teal (*Anas brasiliensis*), Black-bellied Whistling Duck (*Dendrocygna autumnalis*), and White-faced Whistling Duck (*Dendrocygna viduata*; White et al., 1978).

Studies of hematozoa in other regions of the world have identified infections in numerous species of waterfowl, with prevalence rates generally being higher than observed in South America (e.g. Bennett et al., 1981; Cumming et al., 2012; Greiner et al., 1975; Ramey et al., 2012). To date, there have been at least twelve species of haemosporidian parasites identified in waterfowl hosts around the world (Valkiūnas, 2005). Evidence suggests that some of these species may be specific to *Anatidae* hosts (Fallis et al., 1954). Given that hematozoa infections can persist in hosts throughout long distance migrations (Bennett et al., 1991; Valkiūnas, 2005), and some waterfowl species migrate between North America and South America (Botero and Rusch, 1988), it is possible that blood parasite infections could disperse between continents. Thus, waterfowl may

serve as important hosts in the global maintenance of hematozoa parasite diversity.

In this study, our objectives were to: (1) obtain information about the prevalence and geographic distribution of *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* parasites in South American waterfowl; (2) assess the genetic diversity of hematozoa parasites using PCR-based molecular techniques; and (3) compare the phylogenetic relationships among hematozoa haplotypes in South American waterfowl in relation to those previously identified in other South American birds and in North America waterfowl. Our results will enable the assessment of parasite exchange among species and continents, which should be useful for understanding past and potential future shifts in parasite distributions and host ranges.

Materials and Methods

Sample Collection

Whole blood samples were collected from eleven species of South American waterfowl (n = 804) at sites in Peru and Argentina (Figure 1) during dry seasons of 2010 through 2012. Blood samples were collected either from the brachial vein of birds live-captured in mist nets or via cardiac punctures from collected specimens. Samples were immediately frozen in liquid nitrogen and subsequently stored at -80°C until analysis. All capture methods and sampling procedures for this study were reviewed and approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (permit #152985).

Detecting Hematozoa infection

DNA was extracted from all blood samples using the DNeasy Blood and Tissue Extraction Kit (Qiagen, Valencia, California) following the manufacturer's protocol. In order to confirm the viability of each DNA extraction, a 695 base pair (bp) fragment of the mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) gene was amplified using Bird F1 and BirdR1 primers and PCR protocols from Kerr et al. (2007) for all samples except those from *Oxyura jamaicensis*. These primers were unsuccessful in amplifying this fragment of the COI gene in this species, possibly due to the deep divergence from the remaining species sampled (Gonzalez et al., 2009). Therefore, all *O. jamaicensis* samples were verified by amplifying a 529 bp fragment of the COI gene by primers specifically designed for this study (RUDUCOI F2: GTC AAC CAG GAA CTC TTC TAG GG and RUDUCOI R2: GAG ACC CAA TCC TGT ATC AAC AC) and the same protocol used by Kerr et al. (2007). Amplified PCR products for the COI reaction were visualized on 0.8% agarose gels stained with Gel Red Nucleic Acid Gel Stain (Biotium, Hayward, CA).

Each extracted DNA sample that was shown to be viable via our COI positive control was screened for the presence of *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* parasites using a nested PCR protocol described by Hellgren et al. (2004). One negative control was incorporated into each 24-well PCR reaction to ensure absence of contamination, and each sample was screened twice to account for imperfect detection of parasite DNA in host tissue (Ramey et al., 2012). Amplified PCR products were then visualized on 0.8% agarose gels as reported previously.

A target fragment of 479 bp of parasite mtDNA cytochrome *b* gene was bi-directionally sequenced for all samples that were identified as positive for parasite infection to identify parasites by genera and prevent misidentification due to co-amplification (Cosgrove et al., 2006). All samples were treated with ExoSap-it (USB Inc., Cleveland, OH) according to the manufacturers protocol and were not otherwise purified prior to sequencing. Sequencing was conducted using identical primers used for PCR, and BigDye Terminator version 3.1 mix (Applied Biosystems, Foster City, CA) and subsequently analyzed on an Applied Biosystems 3730xl automated DNA sequencer (Applied Biosystems, Foster City, CA). Raw sequence data was cleaned and edited using Sequencher 5.0.1 software (Gene Codes Corp., Ann Arbor, MI). Parasitic infections were then assigned to one of three genera (*Leucocytozoon*, *Haemoproteus*, or *Plasmodium*) using the nucleotide BLAST function available on the National Center for Biotechnology Information (NCBI) website. Assignment of infections was based on the top BLAST score for each sequence that produced a maximum identity score greater than 90% (Ramey et al., 2013). Samples for which double stranded sequences weren't produced from sequencing protocols or could not be assigned via BLAST were considered negative.

Phylogenetic assignment and assessment of haplotype diversity

Diversity of hematozoa mtDNA haplotypes was assessed to determine the relative frequency of parasite haplotypes infecting South American waterfowl, and to establish whether or not there are any shared haplotypes between North America and South America. A median-joining minimum spanning network of parasite cytochrome *b* haplotypes was created using Network 4.6.1 (Bandelt et al., 1999). Additionally, hematozoa

haplotypes infecting South American waterfowl were compared to lineages available on the MalAvi and GenBank public databases (Bensch et al., 2009) as of 27 January, 2014 to determine whether haplotypes closely matched lineages previously identified infecting other avian hosts.

Phylogenetic analyses were performed on hematozoa mtDNA sequence data to verify taxonomic assignment of infections, and to assess evidence for the redistribution of parasites among species and between continents. Phylogenies were constructed by comparing hematozoa mtDNA cytochrome *b* sequence data from South American waterfowl to reference sequences. Reference samples were obtained from NCBI for hematozoa previously reported from passerines in South America, as well as North American waterfowl. *Haemoproteus* and *Plasmodium* mtDNA cytochrome *b* haplotypes identified by Durrant et al. (2006) infecting passerines in Uruguay and Guyana, and *Leucocytozoon* sequences detected in passerines in Peru and Chile obtained from the MalAvi database (Bensch et al., 2009) were included as reference sequences from South America. *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* lineages from ducks and swans sampled in California and Alaska were included as representative sequences from North American waterfowl (Ramey et al., 2012; Ramey et al., 2013). Parasite mtDNA haplotype sequences from South American waterfowl samples identified in this study were included in phylogenetic analyses only if they contained less than three ambiguous bases. All sequences were aligned and cropped to a final length of 358 bp, and haplotypes shorter than this were omitted from phylogenetic analyses. Phylogenies were built using MrBayes 3.2.1 (Ronquist et al., 2012) with a general time reversible model (GTR) and a gamma distribution for among site variation. Four heated chains were used in this analysis, and we

discarded the first 25,000 sampled generations as burn-in. The analysis was replicated multiple times to ensure similar results and each was run for a minimum of 2,000,000 generations or until the split frequencies were less than 0.01, with samples being collected every 1,000 generations. Trees were rooted with mammalian *Plasmodium* cytochrome *b* haplotypes (Genbank accession numbers AF069610, AF069624, AF055587, AY099051, and AY283019) based on methods by Perkins and Schall (2002). Hematozoa mtDNA cytochrome *b* haplotypes generated from this study were submitted to GenBank with accession numbers KJ527070-KJ527081.

Results

Hematozoa detection and prevalence:

Of the 804 waterfowl blood samples screened for hematozoa infection, five samples failed to amplify either the 695 bp or 529 bp region of the COI gene used as positive controls. These samples were dropped from further analyses because we were unable to verify the presence of DNA in these extractions. Of the remaining 799 samples, 25 were positive for infection by *Haemoproteus*, *Plasmodium*, and/or *Leucocytozoon* parasites (3.1%). Two samples were co-infected with parasites of multiple genera for a total of 27 hematozoa infections (Table 1). Eight, five, and fourteen samples were identified as positive for *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* parasites, respectively (Table 1). Of the 25 parasite-positive blood samples, 24 came from hosts collected at field sites in Peru, with only a single positive sample originating from Argentina (Table 1). Infections by *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* parasites were confined to only six of

eleven waterfowl species screened, with the majority of infections identified in Torrent ducks (*Merganetta armata*; Table 1).

Haplotype Diversity:

Analysis of hematozoa mtDNA cytochrome *b* sequences revealed a total of twelve unique haplotypes among South American waterfowl samples (Table 2; Figure 2). Haplotypes were identified as *Plasmodium* (n = 5), *Leucocytozoon* (n = 4), and *Haemoproteus* (n = 3). Only three of the twelve haplotypes detected in our samples occurred in multiple individuals (Haem 1, Plas 3, and Leuc 1; Figure 2). *Haemoproteus* haplotype Haem 1 was identified from two birds of separate species, while *Plasmodium* haplotype Plas 3 was found in six different individuals, all of which were Torrent Ducks. *Leucocytozoon* haplotype Leuc 1 was observed in five different individuals of four species (Table 2).

Eight haplotypes detected in our samples from South American waterfowl were identical to parasite lineages reported on the MalAvi and GenBank databases. *Leucocytozoon* haplotypes Leuc 1 and Leuc 4 were identical to lineages TUSW04 and TUSW05 respectively, which were detected in Alaska Tundra Swans (*Cygnus columbianus*) and California Northern Pintails (*Anas acuta*; Ramey et al., 2012; Ramey et al., 2013; Table 2). South American haplotype Leuc 2 was identical to lineage NOPI04 detected in Northern Pintails in California (Ramey et al., 2013). *Haemoproteus* haplotype Haem 1 was identical to CYGNUS01, which has been previously identified in Tundra Swans in Minnesota and Alaska, Northern Pintails in California, as well as a Mallard (*Anas platyrhynchos*) from

Minnesota (Ramey et al., 2012; Ramey et al., 2013; Ricklefs and Fallon, 2002). Four *Plasmodium* mtDNA cytochrome *b* haplotypes from our samples; Plas 2, Plas 3, Plas 4, and Plas 5 were identical to MILANS05, TFUS05, PESA01, and PADOM11 lineages, respectively. MILANS05 was documented infecting Black Kites (*Milvus migrans*) in Europe (Pérez-Rodríguez et al., 2013), as well as Ruffs (*Philomachus pugnax*) in Africa (Mendes et al., 2013). Lineage TFUS05 was detected infecting Great Thrushes (*Turdus fuscater*) in South America (Lotta et al., 2013). PESA01 was observed in three separate host species: a Pectoral Sandpiper (*Calidris melanotos*) on the Arctic coast of Alaska (Yohannes et al., 2008), a White-tipped Dove (*Leptotila verreauxi*) in Uruguay, and a Mouse-colored Tyrannulet (*Phaeomyias murina*) in Brazil (Durrant et al., 2006; Lacorte et al., 2013). Lastly, the lineage PADOM11 is a common lineage of *Plasmodium*, documented infecting individuals of more than 20 host species in North America, South America, and Asia (e.g. Durrant et al., 2006; Martinsen et al., 2006).

Phylogenetic analysis:

Phylogenetic analysis supported structuring of avian parasite mtDNA haplotypes by genera. For *Haemoproteus* and *Leucocytozoon* parasites, phylogenetic analysis provided strong support for clades comprised of lineages originating from waterfowl sampled in both North and South America, and other clades comprised entirely of parasite mtDNA sequences originating from passerines sampled in South America (Figure 3). Sub-structuring of *Haemoproteus* and *Leucocytozoon* clades by continent of origin was not well supported for lineages detected in waterfowl. Avian *Plasmodium* lineages clustered into two groups of closely related sequences. One group was comprised entirely of haplotypes

originating from parasites infecting waterfowl, and the other consisted of parasite lineages identified in both waterfowl and passerines (Figure 3). Both groups of closely related *Plasmodium* lineages contained sequences from both North America and South America.

Discussion

In our survey of haemosporidian parasites in South American waterfowl, we detected a low level of hematozoa infections among the eleven species sampled (3.1%). The genetic diversity of the parasites we detected included a total of twelve haplotypes infecting six different species of waterfowl. Nucleotide identity comparisons with hematozoa lineages on public databases and phylogenetic analysis of parasite haplotypes provided support for exchange of *Haemoproteus* and *Leucocytozoon* parasites among waterfowl species, and a wider taxonomic range of parasite exchange for *Plasmodium* lineages. Furthermore, we were able to identify identical parasite haplotypes infecting both South American and North American waterfowl, which supports intercontinental exchange of hematozoa by migratory birds.

A review of studies conducted on hematozoa in Neotropical birds by White et al. (1978) reported an overall prevalence of hematozoa among individuals of many avian species to be approximately 10.5%; however, the prevalence of blood parasites may vary seasonally, by habitat types, and among taxa for South American birds. A study conducted in Colombia in 1998 revealed a prevalence rate of 7.8% for *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* parasites among 43 species of birds (Valkiūnas et al., 2003). A survey of 26 bird species across a latitudinal gradient in Chile revealed a prevalence rate of 15.4% for the same three parasite genera (Moreno et al., 2008) and a study of 164 bird species

belonging to 51 families conducted in regions of Guyana and Uruguay observed overall rates of hematozoa prevalence to be 42.1% and 24.2% respectively (Durrant et al., 2006). The low level of hematozoa infection we detected in waterfowl sampled from two regions of South America during the dry season (3.1%) was comparable to apparent prevalence rates for *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* parasites in waterfowl sampled throughout the Neotropics (2.2%) as reported by White et al., (1978). In contrast, a recently published study by Matta et al., (2014), which included the description of a new species, *Haemoproteus macrovacuolatus*, in Black-bellied Whistling Ducks in Colombia reported a 41% prevalence rate for this parasite species in their samples. Previously reported *Haemoproteus* prevalence rates for the same host species were much lower (0.8%). The dramatic difference between prevalence rates was thought to be associated with the different physiological stages of birds that were sampled, as Matta et al., (2014) collected samples from birds during their molting period when they may have a higher probability of being bitten by vectors (Matta et al., 2014).

All positive samples collected from Peru were confined to two sampling locations. Twelve positive blood samples were collected from Lake Titicaca near Puno, and another twelve positive samples were collected along the Rio Chillón north of Lima (Figure 1). Sampling in Lake Titicaca accounted for the majority of our Peruvian samples (n = 353) and was conducted at an elevation of approximately 3824 m. Sampling along the Rio Chillón (n = 129) was done over an elevation gradient ranging from 1092 m to just over 4000 m. Of the 147 samples that were collected at other sites, 124 were collected at locations greater than 1000 m. In previous studies conducted in the Peruvian Andes, the majority of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* infections detected in passerines were from

samples collected between 1000 – 4000 m (Galen and Witt, 2014; Jones et al., 2013).

Therefore, most of our samples were collected at elevations at which we would expect hematozoa prevalence to be highest.

Blood samples collected from Argentina produced only one sample detected as positive for parasite infection (Table 1). The habitats in Argentina from which our samples were collected tended to be arid and very windy, which may explain the low apparent parasite prevalence. The species of haemosporidian parasites investigated in this study are all transmitted by blood sucking insect vectors that have aquatic and flighted life stages, both of which may be impeded by adverse climatic conditions at sampling locations. Previous studies on avian hematozoa conducted in Argentina have produced results showing either very low prevalence rates or a complete lack of blood parasite infection in wild birds (D'Amico et al., 2008; Masello et al., 2006).

Out of all the zoogeographic regions in which avian haemosporidians have been documented infecting wild birds, the Neotropics have the second lowest species diversity of avian hematozoa next to the Australian region (Valkiūnas, 2005). Of the three genera of parasites examined in this study, only *Plasmodium* has historically been observed to have similar levels of species diversity in the Neotropics compared to other zoogeographic regions. In concordance with previous studies, we found more *Plasmodium* haplotypes in South American waterfowl than either *Haemoproteus* or *Leucocytozoon*.

The prevalence of infections by *Leucocytozoon* parasites has historically been reported to be low in wild birds sampled throughout South America (e.g. Bennett et al., 1991; Bennett and Borrero, 1976; Matta et al., 2004; Rodríguez and Matta, 2001). This may

be attributed to a lack of viable vectors for transmission of *Leucocytozoon* spp., since numerous species of birds breeding in North America overwinter in Neotropical regions and may act as carriers for parasites between continents (Bennett et al., 1991; Rodríguez and Matta, 2001). The only species of *Leucocytozoon* reported to infect *Anatidae* waterfowl is *Leucocytozoon simondi*, which has been reported to be endemic to Holarctic regions and isn't known to be successfully transmitted below 42° North latitude (Valkiūnas, 2005). The detection of four *Leucocytozoon* haplotypes infecting individuals of four separate species of waterfowl in Peru (Table 2; Figure 2), including three species endemic to South America, provides evidence that *Leucocytozoon simondi* parasites are transmitted in South America among waterfowl species. Morphological examination of blood smears made from South American endemic waterfowl is necessary to identify if the morphospecies of *Leucocytozoon* parasites infecting ducks in Peru is *L. simondi* or another species. Close genetic relationships among *Leucocytozoon* haplotypes from waterfowl sampled in both North America and South America suggest the former.

Our query of parasite lineages reported in public databases and phylogenetic analysis supports haplotype sharing for *Plasmodium* parasites among continents and diverse avian taxa. Additionally, these analyses support gene exchange of *Leucocytozoon* and *Haemoproteus* haplotypes among species of North American and South American waterfowl. Although species and subspecies of waterfowl from which *Leucocytozoon* and *Haemoproteus* parasites were detected in our study are not reported to migrate between North America and South America, other species including Blue-winged Teal (*Anas discors*) breed in North America and winter in the Neotropics (Botero and Rush, 1988), which may facilitate the dispersal of hematozoa parasites between continents.

The lack of identical *Haemoproteus* and *Leucocytozoon* lineages on public databases from non-waterfowl hosts and phylogenetic divergence between parasite haplotypes previously detected in South American passerines and those from waterfowl supports some degree of host-specificity for *Haemoproteus* and *Leucocytozoon* lineages detected in this study. Previous research has found evidence that *Haemoproteus* parasites exhibit higher levels of host specificity when compared to *Plasmodium* species (Bensch et al., 2000; Krizanauskiene et al., 2009), but less work has been conducted to assess the specificity of *Leucocytozoon* parasites. Fallis et al. (1954) attempted to experimentally infect wild and domestic hosts with *L. simondi* including ducks, geese, grouse, chickens, turkeys, and pheasants. However, infections only developed in ducks and geese, which suggest that this species may be specific to waterfowl.

Our investigation extends the available data for hematozoa infections in South American waterfowl and suggests that additional studies are warranted to better understand the ecology of hematozoa in Neotropical birds. Sampling a range of host species including passerines, waterfowl, and other taxa at specific locations through time could provide important information for further assessment of parasite exchange and seasonality of transmission. Contrasting parasite screening results from samples collected during wet and dry seasons, on either side of geographic barriers such as the Andes, and across an elevation gradient could help inform how ecological factors affect parasite prevalence rates. Further genetic characterization of *Leucocytozoon* infections, paired with microscopic examination of blood smears, would reveal what morphospecies are infecting these South American waterfowl. Lastly, further investigation into host and regional patterns of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* parasite infections in South

American birds may provide further inference into potential for shifts in geographic and host ranges.

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- 1) Huacho (n = 23)
- 2) Rio Chillon (n = 129)
- 3) Junin (n = 66)
- 4) Apurimac (n = 58)
- 5) Puno (n = 353)
- 6) Buenos Aires (n = 18)
- 7) Baradero (n = 15)
- 8) Bahia Bustamante (n = 28)
- 9) Sarmiento (n = 18)
- 10) Rio Malargue (n = 28)
- 11) Valle Hermoso (n = 17)
- 12) Arroyo Grande (n = 46)

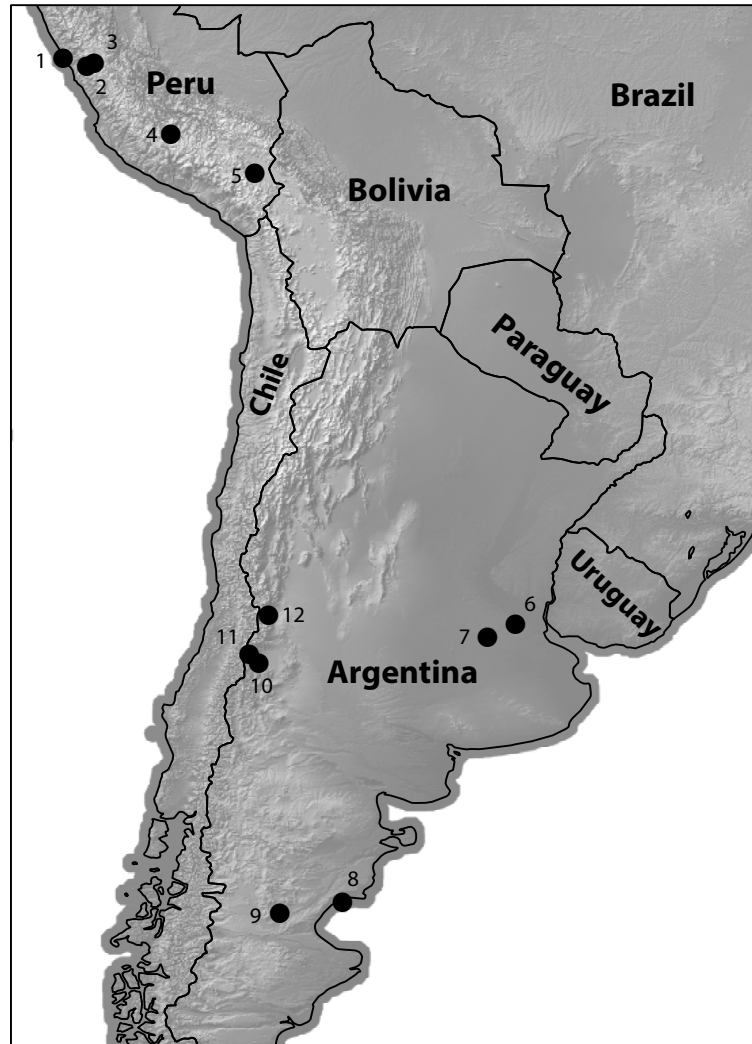


Figure 1: Map of sampling locations in Peru and Argentina. The number of waterfowl blood samples collected at each site is provided in parentheses.

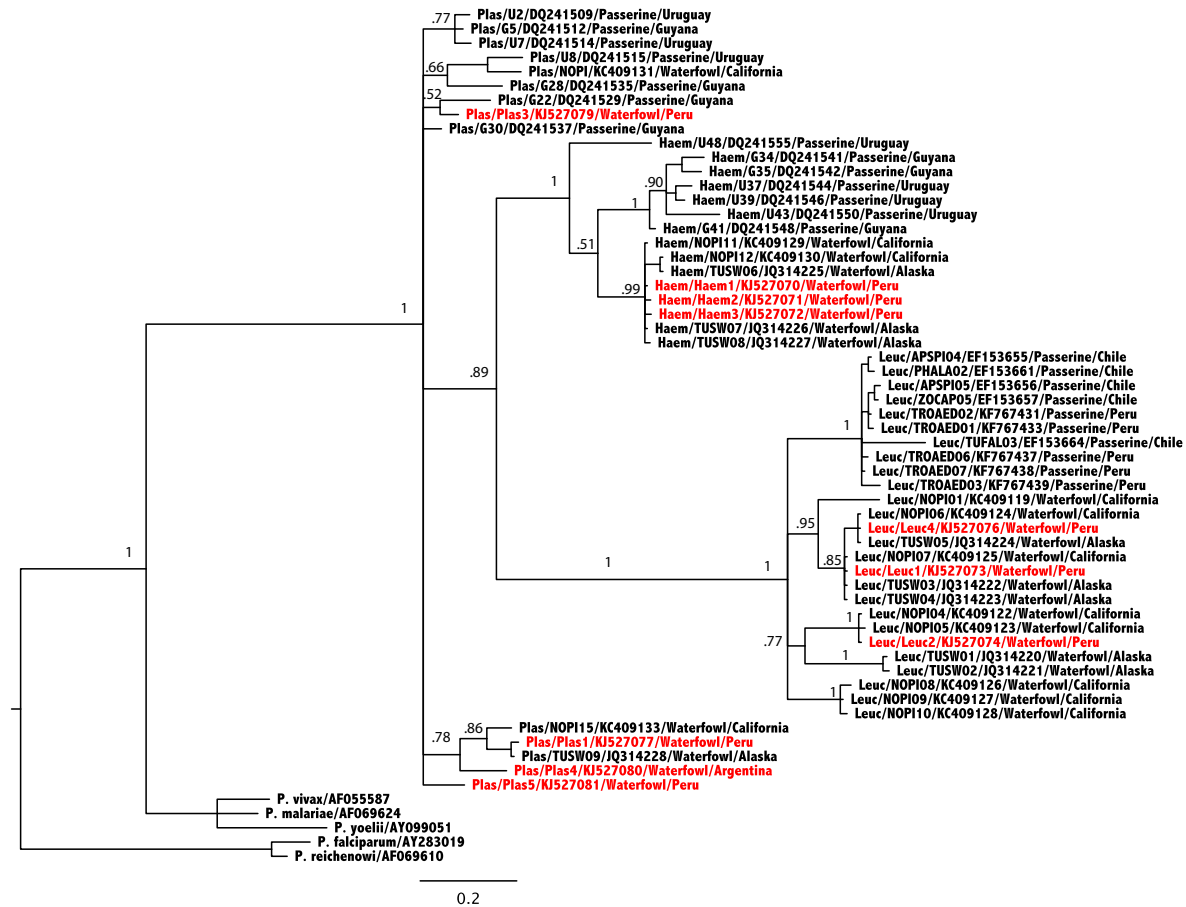


Figure 3: Bayesian phylogenetic tree of hematozoa mitochondrial DNA cytochrome *b* haplotypes obtained from infected waterfowl. Trees were rooted with mammalian *Plasmodium* outgroups. Node tips are labeled with parasite genus (Haem = *Haemoproteus*, Leuc = *Leucocytozoon*, and Plas = *Plasmodium*), followed by the lineage name, Genbank accession number for each sequence, host order (passerine/waterfowl), and the country/state from which the samples were collected. All haplotypes identified in this study are highlighted in red. Numbers on branches represent posterior probabilities from the analysis. All reference sequences were obtained from the National Center for Biotechnology Information website.

Table 1: Number of blood samples from South American waterfowl detected as positive for *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* parasites by country and species.

Sample origin (Country and Species)	Samples tested	<i>Leucocytozoon</i> positive	<i>Haemoproteus</i> positive	<i>Plasmodium</i> positive
Peru				
<i>Anas bahamensis</i>	3	0	0	0
<i>Anas cyanoptera</i>	77	3	2	1
<i>Anas flavirostris</i>	65	1	0	0
<i>Anas georgica</i>	49	0	1	0
<i>Anas puna</i>	92	0	0	0
<i>Chloephaga melanoptera</i>	30	0	0	0
<i>Lophonetta specularioides</i>	40	0	0	0
<i>Merganetta armata</i>	177	1	0	12
<i>Oxyura jamaicensis</i>	96	3	2	0
Total	629	8	5	13
Argentina				
<i>Anas bahamensis</i>	2	0	0	0
<i>Anas cyanoptera</i>	6	0	0	0
<i>Anas flavirostris</i>	15	0	0	0
<i>Anas georgica</i>	26	0	0	0
<i>Anas versicolor</i>	4	0	0	0
<i>Dendrocygna bicolor</i>	1	0	0	1
<i>Lohponetta specularioides</i>	50	0	0	0
<i>Merganetta armata</i>	66	0	0	0
Total	170	0	0	1

Table 2: Hematozoa mitochondrial DNA cytochrome *b* haplotypes detected in South American waterfowl species and results of comparison to previously identified parasite lineages as listed on the MalAvi and GenBank databases.

Haplotype	Host species	MalAvi/GenBank Lineage	Identity Score MalAvi/GenBank
Haem 1	<i>A. cyanoptera</i> , <i>O. jamaicensis</i>	Cygnus01/TUSW07	100% 100%
Haem 2	<i>O. jamaicensis</i>	Cygnus01/TUSW07	99% 99%
Haem 3	<i>A. georgica</i>	Cygnus01/TUSW07	99% 99%
Leuc 1	<i>A. cyanoptera</i> , <i>A. flavirostris</i> <i>O. jamaicensis</i> , <i>M. armata</i>	TUSW04/ TUSW04	100% 100%
Leuc 2	<i>O. jamaicensis</i>	HELLI02/NOPI04	97% 100%
Leuc 3	<i>O. jamaicensis</i>	HELLI02/NOPI04	96% 98%
Leuc 4	<i>A. cyanoptera</i>	TUSW05/TUSW05	100% 100%
Plas 1	<i>A. cyanoptera</i>	BT7	99% 99%
Plas 2	<i>M. armata</i>	MILANS05	100% 100%
Plas 3	<i>M. armata</i>	TFUS05	100% 100%
Plas 4	<i>D. bicolor</i>	PESA01	100% 100%
Plas 5	<i>M. armata</i>	PADOM11	100% 100%

CHAPTER 2

A real-time, quantitative PCR protocol for assessing the relative parasitemia of *Leucocytozoon* in waterfowl¹

Abstract: Microscopic examination of blood smears can be effective at diagnosing and quantifying hematozoa infections; however, this method requires highly trained observers, is time consuming, and may be inaccurate for detection of infections at low levels of parasitemia. In an effort to create a novel methodology for identifying and quantifying *Leucocytozoon* parasite infection in wild waterfowl species (Anseriformes), we designed a real-time, quantitative PCR protocol to quickly and efficiently amplify *Leucocytozoon* mitochondrial DNA using TaqMan fluorogenic probes and validated our methodology using blood samples collected from waterfowl in interior Alaska during late summer and autumn ($n = 105$). By comparing our qPCR results to those derived from a widely used nested PCR protocol, we determined that our assay showed high levels of sensitivity (91%) and specificity (100%) in detecting *Leucocytozoon* DNA from host blood samples. Additionally, linear regression showed significant correlation between low Ct values, the raw measure of parasitemia produced by our qPCR assay, and greater numbers of parasites observed on blood smears ($R^2 = 0.67$, $P = 0.003$), providing evidence that our assay is useful for determining relative parasitemia rates among samples. This methodology provides a

¹ Smith, M.M., Schmutz, J., Apeltgren, C., and Ramey, A.M. 2014. A real-time, quantitative PCR protocol for assessing the relative parasitemia of *Leucocytozoon* in waterfowl. Prepared for submission to *Experimental Parasitology*

powerful new tool for studies assessing effects of haemosporidian infection in wild avian species.

Keywords: *Leucocytozoon*; parasitemia; qPCR; waterfowl

Introduction:

Detection and quantification of parasite infection levels (parasitemia) in avian hosts is an important component of research assessing pathological effects of parasite infection and host responses. While numerous studies have assessed mortality as a response of haemosporidian infection (Bueno et al., 2010; Cellier-Holzem et al., 2010; Donovan et al., 2008), sub-lethal effects have not been well studied. Experimental infection of avian species has shown that pathogenicity of haemosporidian parasites strongly depends on levels of infection (Palinauskas et al., 2008). Low level, chronic infections with *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* parasites have been shown to be relatively benign to host in a variety of species (Valkiūnas, 2005), while high, acute levels can cause anemia, decreased hematocrit values, and hypertrophy of the liver and spleen (Ots and Hõrak, 1998; Palinauskas et al., 2008). Although previous studies on the effects of parasitemia levels in avian hosts have relied on experimentally infected domestic species (e.g. Cellier-Holzem et al., 2010; Zehtindjiev et al., 2008), few studies have examined effects of varying levels of blood parasite infection in wild organisms (e.g., Knowles et al., 2010b) which might be useful for understanding sub-lethal effects on free-ranging hosts.

Historically, hematozoa infections have been studied through microscopic examination of peripheral blood smears, allowing the examiner to simultaneously diagnose blood parasite infections, and quantify parasitemia levels. Although microscopic examination of blood films is time-tested and relatively inexpensive, it can also be time consuming and requires highly trained individuals to correctly identify haemosporidians infecting host erythrocytes (Waldenström et al., 2004). In recent years, molecular methods based on polymerase chain reaction (PCR) techniques have increasingly been used to assess hematozoa prevalence. These methods have the advantage of allowing researchers to test large numbers of samples quickly for the presence of blood parasites, and to obtain parasite genetic data through DNA-sequencing of PCR-amplified products. However, PCR-based methods that use parasite mitochondrial DNA (mtDNA) to screen for presence or absence of parasite infection give no measure of the level of infection in each host, and these methods commonly underestimate mixed infections by amplifying the parasite species with the highest parasitemia (Valkiūnas et al., 2006). Several studies have compared the effectiveness of molecular screening methods to microscopic examinations. Valkiūnas et al., (2008) found results of PCR diagnostics to be similar to those produced by microscopy, while other studies have reported that microscopy had reduced detection sensitivity, particularly when parasitemia was very low (e.g., Alexander et al., 2002).

The application of real-time, quantitative PCR (qPCR) methods to the field of haemosporidian research has provided an opportunity for evaluation of parasitemia levels in avian blood samples, previously only possible through microscopic examination of blood smears, while maintaining many of the advantages of molecular screening approaches (e.g. increased sensitivity, high throughput, and less observer bias). These quantitative methods

are based on the ability to monitor the amplification of a specific target sequence in real time, through the use of fluorescent probes or DNA-binding dyes (Bell and Ranford-Cartwright, 2002). Several studies have recently developed real-time, quantitative PCR (qPCR) protocols for determining levels of infection by *Haemoproteus* and *Plasmodium* species in passerine birds (e.g. Cellier-Holzem et al., 2010; Friedl and Groscurth, 2011; Knowles et al., 2010a), but an optimized and validated qPCR methodology specific to *Leucocytozoon* parasite species has not yet been reported.

We present a methodology to quickly and accurately quantify the amount of *Leucocytozoon* DNA present in avian blood samples using qPCR. High prevalence rates of *Leucocytozoon* infections have been observed in populations of anseriform waterfowl, particularly those that live or breed in temperate northern regions of the Holarctic (e.g., Ramey et al., 2012; Valkiūnas, 2005). Although *Leucocytozoon* parasite infection isn't known to cause mortality in wild waterfowl, studies that examined sub-lethal effects of *Leucocytozoon* infection in other wild bird species have found detrimental effects on host reproductive success and parental care (Merino et al., 2000; Wiehn et al., 1999). An optimized and validated method to quickly determine a host's relative parasitemia level might allow researchers to screen many samples from a population to better assess negative effects of *Leucocytozoon* infection.

Materials and Methods:

Development of quantitative PCR assay

Numerous strategies have been developed to quantify DNA in real time (Bell and Ranford-Cartwright, 2002). The two most popular methods for use in pathogen detection are based on the non-specific, double-stranded DNA (dsDNA) intercalator, SYBR Green®, and TaqMan® fluorogenic probes. We chose to use TaqMan probes in our qPCR assay as they can be designed to be highly specific to a DNA target region (see Heid et al., 1996). This method is likely beneficial when attempting to amplify specific regions of parasite DNA in the presence of large amounts of host DNA or mixed haemosporidian infections. In contrast, SYBR Green dyes bind to all dsDNA, which may be problematic when attempting to design a genus-specific assay that only amplifies *Leucocytozoon* DNA.

Because previous work in interior Alaska has shown moderate to high prevalence rates of *Haemoproteus* and *Plasmodium* parasites in addition to *Leucocytozoon* (Reeves et al., In review), our goal was to design genus-specific primers that would efficiently amplify our target region of *Leucocytozoon* DNA without amplifying DNA from *Haemoproteus* or *Plasmodium* parasites present in blood samples. To accomplish this, we obtained *Leucocytozoon* mtDNA cytochrome *b* (cyt-*b*) gene sequences described as infecting multiple avian host species from GenBank, as well as homologous *Haemoproteus* and *Plasmodium* mtDNA cyt*b* sequences, also from multiple host species, and aligned them using Sequencher 5.0.1 software (Gene Codes Corp., Ann Arbor, MI). Using this alignment, we designed multiple primer/probe combinations (Table 1) using the Custom Gene Expression Assay Design Tool (www.lifetechnologies.com). Target regions were selected in areas of

the *cytb* gene that were conserved across *Leucocytozoon* lineages but divergent between *Haemoproteus/Plasmodium* and *Leucocytozoon*.

Sample collection & blood smear preparation

Whole-blood samples were collected from seven species of waterfowl at two locations in interior Alaska during the late summer and fall of 2013. Blood samples were collected from birds captured on the Chena River in Fairbanks ($n = 70$) using whoosh nets and swim-in traps from August to October (UAF IACUC #358515), and hunter-harvested birds were collected from the Minto Flats State Game Refuge ($n = 35$) during September. Whole blood was collected from the jugular vein of live birds and obtained via cardiac punctures from hunter-harvested individuals. Samples were immediately preserved in lysis buffer solution (Longmire et al., 1988) and subsequently stored at -80°C until analysis. Additionally, a small amount of each blood sample was used to create peripheral blood smears for microscopic analysis of parasitemia levels. Two smears were prepared for each sample on ready-to-use, clear-glass slides and air dried immediately after preparation. All smears were fixed in absolute methanol for 1 min on the same day of their preparation and were later stained using dif-Quik staining solution (Polysciences Inc., Warrington, PA).

DNA Extraction & Nested-PCR Analysis

Genomic DNA was extracted from all blood samples using the DNeasy Blood and Tissue Extraction Kit (Qiagen, Valencia, California) following the manufacturer's protocol.

To verify that each extraction was successful, a 695 base pair (bp) fragment of host mtDNA cytochrome oxidase I (COI) gene was amplified following protocols in Ramey et al. (2013). Aliquots of 100 µl of each extraction were used in the development of our qPCR assay. Total DNA concentration in these aliquots was measured using a Quant-it assay (Life Technologies, Grand Island, NY) and each extraction to be used for qPCR analysis was diluted to a concentration of 5 ng/µl.

To obtain DNA sequence information for each infection and evaluate the sensitivity and specificity of our qPCR protocol, we screened each sample using a nested PCR method described by Hellgren et al. (2004) to test for *Leucocytozoon* infection. This protocol was conducted on each sample until clean, double stranded sequence of the 479 bp target region was obtained or the protocol was run five times if samples seemed to be negative for *Leucocytozoon* infection. Additionally, each sample was screened for the presence of *Haemoproteus* and *Plasmodium* infection to assess whether subsequent qPCR procedures amplified non-specific DNA. Post-PCR procedures for PCR cleanup and DNA sequencing followed those used by Ramey et al. (2013).

Application of qPCR Assay

After testing all primer and probe sets (Table 1), we chose to use LEUC_02F (5'-GTT ACT TAC CTT TAT CAT GGA GTA GTG GTT T-3') and LEUC_02R (5'-CTC ATT TGA CCC CAT GGT AAG ACA T-3') in combination with a dual-labeled TaqMan probe (5'-CCC ATG AAA GCA GTT ACA ATA-3') which amplify a 97 bp (including primers) target region of parasite

cytb gene. This primer/probe set consistently produced the best amplification curves (see Caraguel et al., 2011a) and reaction efficiency when run simultaneously with other primer/probe combinations. Each 20 µl reaction contained 5 µl of template DNA, 0.5 µl of 20X TaqMan primer/probe combination, 10 µl of IQ supermix (Bio-rad) containing dNTP's and hot-start iTaq DNA polymerase, and 4.5 µl of nano-pure water. PCR reactions were performed using 96-well plates on a CFX96-Connect real-time system (Bio-rad). The temperature profile included a denaturation period of 8 minutes at 95 ° C, followed by 45 cycles of 95 ° C for 15 seconds and 60° C for 60 seconds.

To produce *Leucocytozoon* DNA standards, we amplified the 479 bp region of *Leucocytozoon cytb* gene that is targeted by the nested-PCR protocol designed by Hellgren et al. (2004). Total DNA concentration of this amplification was quantified using the same Quant-it assay as for all other extractions, and we produced a six step, 10-fold dilution series from this initial DNA product ranging from 5.0×10^{-3} ng/µl (standard 1) down to 5.0×10^{-8} ng/µl (standard 6), corresponding to a range of 9.67×10^6 –96.7 copies of our target segment of parasite *cytb* gene. Each sample was run in triplicate, and resulting cycle threshold (Ct) values, or the first cycle with a detectable fluorescence above background noise, were averaged across the three wells. To ensure that the cycle threshold was consistent across all reactions, we manually set the threshold for our first standard curve, representing 5.0×10^{-3} ng/µl of parasite mtDNA, to a Ct of 16 for every 96-well run. To ensure repeatability and consistency of our Ct results, we re-extracted DNA from a sub-set of 25 samples that were used for microscopic analysis and re-ran them to determine if there would be any significant differences between the mean Ct values of each run.

The validity of each sample's results was based on three criteria: 1) if the efficiency of each reaction, calculated as $E=10^{(-1/\text{slope})}$, was between 95%–105%. Efficiency above or below this range could indicate problematic amplification of target regions (Reynisson et al., 2006); 2) R^2 values for the slope of the standard curve was >0.99 as a lower value could indicate inefficient amplification of the standards (Friedl and Groscurth, 2011); 3) the standard deviation between all three replicates of each sample was less than 1.0. If samples showed a standard deviation greater than 1.0, they were re-run on separate reaction plates to determine if the original high variation was due to laboratory error (Friedl and Groscurth, 2011). If the second run of the samples also produced standard deviations greater than 1, those samples were removed from analysis. Additionally, results from any reactions that produced efficiency or R^2 values that violated either of the first two criteria were thrown out and the sample re-run.

Microscopic Analysis

To ensure that our qPCR results could be compared accurately to parasitemia levels, we quantified the level of *Leucocytozoon simondi* infection in each sample using microscopic examination. A sub-set of 25 smears was independently quantified by two individuals (MS and CA), the latter being a blind observer in order to eliminate the potential bias caused by knowing whether each sample was infected with *L. simondi* based on nested PCR results. One hundred fields were observed for each blood smear under 400X magnification, and each slide that was negative after 100 fields was also scanned further to determine whether low-level infections were missed. Parasites that were detected in the

fields were counted to quantify parasitemia levels and each sample was considered either positive or negative based on the summation of all slide-reading efforts (i.e. 100 fields and scanning efforts outside of fields). Parasites that were detected outside the 100 fields during the broad range scanning, however, were not counted towards parasitemia estimates. A digital photograph was taken of every tenth field to estimate the number of red blood cells (RBCs) observed for each slide. The total number of RBCs in each photograph were counted manually and with a custom ImageJ program (Gering and Atkinson, 2004). The average number of RBCs was calculated across all ten photographs and this number multiplied by 100 to produce an estimate for the total number of RBCs examined in all fields.

Statistical Analyses

We examined two different scales of microscopy data for examining the relationship between number of parasites observed on slides and the Ct values produced by qPCR analysis. For a higher resolution comparison, we calculated the average parasitemia rate (number of observed host RBCs infected by *Leucocytozoon* parasites divided by the estimated total number of RBCs examined) for each sample per observer, and then averaged the two rates. We then used this average value (Average Rate) as an independent variable in a linear regression model of how well Ct values related to ocular detection of parasites on slides. We developed another somewhat similar independent variable (Average Detects) for this regression model using data derived from total slide-reading efforts. If neither observer detected any parasites in either the 100 fields used for

estimating parasitemia rates or a subsequent broad-scale scan, then this variable was recorded as 0 for a given sample. If one of the two observers detected one or more parasites during slide reading efforts, then this variable was recorded as 1, and if both observers detected one or more *Leucocytozoon* parasites then this variable was recorded as 2.

Our linear regression model thus related the Average Rate of parasitemia and the Average Detection of parasites to the average Ct value for each sample using a least-squares modeling approach in SAS (SAS Institute, 2002). Because our data contained many zeros and were not normally distributed, we used a randomization approach for evaluating the statistical significance of our models and a bootstrapping approach for estimating the means and standard errors of model parameters (Manly, 1997). Because we were examining two independent variables, we also used an information-theoretic approach (Burnham and Anderson, 2002) to evaluate whether models with one independent variable were more parsimonious than our pre-conceived model with two variables. In this statistical approach, smaller AIC values connote a better, more parsimonious model. To test whether there were significant differences between the mean Ct values of our original DNA extractions and re-extractions of the same samples, we conducted a paired t-test between the two data sets using the program R (R Core Team, 2013).

Results

qPCR Results

Of the 105 samples screened using our qPCR assay, seven failed to produce results with standard deviations <1.0 between three replicates even with repeated runs and thus results for these samples were dropped from further analysis. The remaining 98 samples produced viable, quantitative results with Ct values falling between 26 and 41. Based on the resulting Ct values of our standard curves, corresponding gene copy numbers of each of those standards, and results from our statistical analysis, we determined our Ct cutoff to be cycle number 35 (see (Caraguel et al., 2011)). Samples that amplified past this point were considered to be negative as gene copy numbers at that point would theoretically be <10 and may be indistinguishable from thermal degradation of probes or fluorescent artifacts (Caraguel et al., 2011).

Using a Ct cutoff of 35, our method showed high levels of sensitivity and specificity for identifying *Leucocytozoon* infection from waterfowl blood samples. We were able to successfully amplify parasite DNA in 92% of samples shown to be positive by nested-PCR analysis and DNA sequencing (Table 2). In addition, all samples that tested negative for *Leucocytozoon* spp. infection after five replicate reactions of nested-PCR all had Ct values >35 using our qPCR protocol, indicating an assay specificity of 100% (Table 2).

Microscopy Results

Quantification of infected erythrocytes in blood smears was difficult due to the low parasitemia rates of our samples. Of the 25 slides that were examined, 11 were diagnosed as positive by one or both observers (Table 3) for *Leucocytozoon simondi* infection based on the 100 observed fields and/or broad scanning outside fields. Eight samples produced low parasitemia rates (Figure 1; Table 3) and all remaining smears from the sub-set of 25 examined for correlation with qPCR results were diagnosed as negative for *Leucocytozoon* infection using microscopy. In comparison, double-stranded *Leucocytozoon* mtDNA was amplified using nested PCR from 20 of 25 paired samples examined via microscopy and 19 of the 20 nested PCR positive samples had Ct values < 35 (Table 3).

Statistical Results

The statistical model with Average Rate and Average Detection of blood parasites on slides was significantly correlated with Ct values ($R^2 = 0.694$, $P = 0.003$; Figure 1). This model was superior to those with Average Rate only (8.436 AIC units greater; Table 4) or Average Detection only (3.704 AIC units greater; Table 4). The intercept parameter ($\beta = 35.143$, $SE(\beta) = 0.498$) implies that Ct values greater than 35.1 signify blood samples with no detectable parasitemia. Both regression coefficients ($\beta = -319$, $SE(\beta) = 240$ for Average Rate; $\beta = -1.715$, $SE(\beta) = 0.463$ for Average Detection) demonstrate that greater numbers of parasites observed on slides was correlated with higher Ct values (Figure 1). Additionally, the paired *t*-test between original DNA extractions for our 25 sample subset and re-extractions showed no significant difference between Ct values with a mean

difference between extractions of the same sample of 0.5467 ($t = 1.8016$, $df = 20$, $p\text{-value} = 0.08671$).

Discussion

The qPCR assay developed for this study was successfully used to identify *Leucocytozoon* parasite infections and determine relative parasitemia rates in Alaskan waterfowl samples. Sensitivity of our qPCR assay was similar to a widely used nested PCR assay and specificity was high, even in the presence of mixed infections of *Haemoproteus* and *Plasmodium*. Results from microscopic examination of blood smears correlated well with qPCR results, validating the ability of our assay to provide a relative quantitative measure of parasitemia using molecular methods.

A potential limitation of our efforts to validate the qPCR assay described here is temporal bias of our sample collection. As blood samples smears were all collected from wild birds in the late summer and autumn in interior Alaska, during which time *Leucocytozoon* transmission may be limited and most infections are presumably in a chronic stage, most of our samples likely represent low level infections (Valkiūnas, 2005). Hematozoa infections such as these can present as low as 1/1,000,000 infected erythrocytes and are easily miss-diagnosed as negative by microscopic examination of blood smears (Zehtindjiev et al., 2008). Furthermore, previous work has indicated that obtaining samples from wild avian species that possess high parasitemia levels through traditional capture methods can be very difficult due to decreased mobility and altered behavior of heavily parasitized individuals (Valkiūnas, 2005). Thus, our results showing

much higher sensitivity of our qPCR assay as compared to microscopy could be related to low levels of parasitemia in our samples. Additionally, other studies have indicated that accuracy of qPCR assays diminishes in samples with heavy parasitemia levels (>10% infected erythrocytes), while microscopic methods are very effective at quantifying infections in samples with these high parasitemia rates. Thus, we encourage validation of this assay for investigations reporting on high level of *Leucocytozoon* infections in waterfowl to ensure rigorous inference regarding relative levels of parasitemia in waterfowl samples regardless of stage or level of infection.

Several studies that have produced similar real-time, quantitative PCR protocols targeting *Haemoproteus* and *Plasmodium* species in avian hosts have extrapolated their resulting Ct values into estimated parasite gene copy number, percent parasitemia, or even the estimated number of parasites per 100 erythrocytes (Bentz et al., 2006; Cellier-Holzem et al., 2010; Friedl and Groscurth, 2011). Because the goal for this study was to provide an optimized protocol for assessing the relative parasitemia for a given sample set, we chose to report our results as raw Ct values rather than transform them into other derived estimates. Looking at the range of Ct values produced by our assay (26-35), it is clear that some individuals carried higher parasite loads at the time of sampling as each 3.52 cycle change indicates a 10-fold increase in the starting number of parasite DNA concentration.

A benefit of using TaqMan probes for real-time PCR analysis is the ability to run multiplex qPCR reactions, allowing researchers to quantify fluorescence from multiple primer/probe sets simultaneously in a single reaction well (Bell and Ranford-Cartwright, 2002; Schneider et al., 2005; Veron et al., 2009). Thorough validation of such a multiplexed

assay could eventually eliminate the need to use the nested PCR diagnostic approach for avian hematozoa research in specific instances where parasitemia data is of interest and genetic characterization of parasites is not necessary to achieve project goals. The development and incorporation of new primers/probes specific to *Haemoproteus* and *Plasmodium* parasite DNA and amplification and dilution of new standard curves would be a valuable contribution towards this goal.

The incorporation of quantitative measures of parasitemia into research efforts to assess fitness consequences of hematozoa infections may allow for more refined inference as compared with nested PCR analysis only, as qPCR provides additional information on the level of infection in each sample. However, specific project goals and assay costs should be considered when determining project methodology as, aside from being diagnostic, nested PCR and qPCR provide different types of data. With such information, researchers could incorporate the level of infection as a variable in inference models that seek to explain variation in survival, nesting success, body condition, or migration timing. Lastly, the process used in this study by running samples through the nested protocol by Hellgren et al. (2004) to determine positive samples and then using separate DNA extraction aliquots to quantify parasitemia rates in those samples appears to be the most logical and efficient way to obtain DNA sequence data of parasites and also quantify the level of infection in each sample.

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Table 1: Summary of primer/probe sets designed for amplification of *Leucocytozoon* mtDNA *cytb* gene regions. All primer and probe sequences are oriented in the 5'– 3' direction and amplicon lengths include primers.

Primer Set	Forward Primer	Reverse Primer	Probe Sequence	Amplicon Length
Leuc_01	CTGCTTTCATGGGT	AAGTGTAATACAAAG		160 bp
	TATGTCTTACCA	AATCTTTTTTAATGTT	AATCCACCACAA	
		GGATCATTAATAATA	ACCC	
Leuc_02	GTTACTTACCTTTA			97 bp
	TCATGGAGTAGTGG	CTCATTTGACCCCAT	CCCATGAAAGCA	
	TTT	GGTAAGACAT	GTTACAATA	
Leuc_03	ATTAATGATCCAAC	AGGATTAGTGCTACC		116 bp
	ATTAAAAAGATTCT	TTGAATATGTAAGAA	TTCCCATTCGTA	
	TTGTATTACACTTT	GA	GCTTTAG	

Table 2: Summary of results from nested and qPCR analysis of *Leucocytozoon* infection of blood samples from Alaskan waterfowl.

Nested PCR	TaqMan qPCR	No. of Samples (Total = 98)
Pos	Pos	71
Pos	Neg	7
Neg	Pos	0
Neg	Neg	20

Table 3: Results of nested-PCR, qPCR, and microscopic examination of blood smears from 25 samples. qPCR pos/neg was determined by whether or not amplification occurred below cycle 35. Each sample was considered pos/neg by each observer during microscopic examination if parasites were observed in any of 100 fields or during broad screening outside of fields. Only parasites observed within the 100 fields were counted towards parasitemia estimates.

Sample	Nested PCR pos/neg	qPCR pos/neg	Ct value	Blind observer pos/neg	Blind observer % parasitemia	Unblind observer pos/neg	Unblind observer % parasitemia
MMS13003	+	+	33.62	-	0	-	0
MMS13007	+	+	29.98	+	0	+	0.00655
MMS13009	+	+	34.80	-	0	-	0
MMS13010	+	+	30.92	-	0	+	0.00275
MMS13012	+	+	31.73	+	0	+	0
MMS13014	+	+	34.17	-	0	+	0.00199
MMS13016	+	+	29.28	+	0.01008	+	0.00833
MMS13017	+	+	34.82	-	0	-	0
MMS13018	+	+	26.95	+	0.02137	+	0.01589
MMS13021	+	+	34.70	+	0	-	0
MMS13043	-	-	38.43	-	0	-	0
MMS13044	+	-	36.44	-	0	-	0
MMS13058	+	+	33.91	-	0	-	0
MMS13059	+	+	34.38	-	0	-	0
MMS13060	-	-	37.87	-	0	-	0
MMS13062	+	+	32.05	-	0	-	0
MMS13063	+	+	32.75	-	0	+	0
MMS13064	+	+	32.69	-	0	+	0.00236
MMS13065	+	+	31.89	+	0	+	0.00396
MMS13066	+	+	32.15	-	0	-	0
MMS13067	+	+	33.74	-	0	-	0
MMS13089	-	-	36.80	-	0	-	0
MMS13090	-	-	36.43	-	0	-	0
MMS13094	-	-	36.95	-	0	-	0
MMS13097	+	+	30.88	+	0.00323	+	0.00227

Table 4: Summary of model parameters and resulting AIC values from linear regression analysis. Comparison was between Ct values produced by qPCR, and parasitemia estimates from microscopic analysis of blood smears.

Model	AICc	ΔAICc	AIC calc	AICc weight
AvgRate + AvgDetects	29.751	0	1	0.853
AvgDetects	33.455	3.704	0.156923006	0.134
AvgRate	38.187	8.436	0.014728071	0.013
Null	103.326	73.575	1.05534E-16	0.000

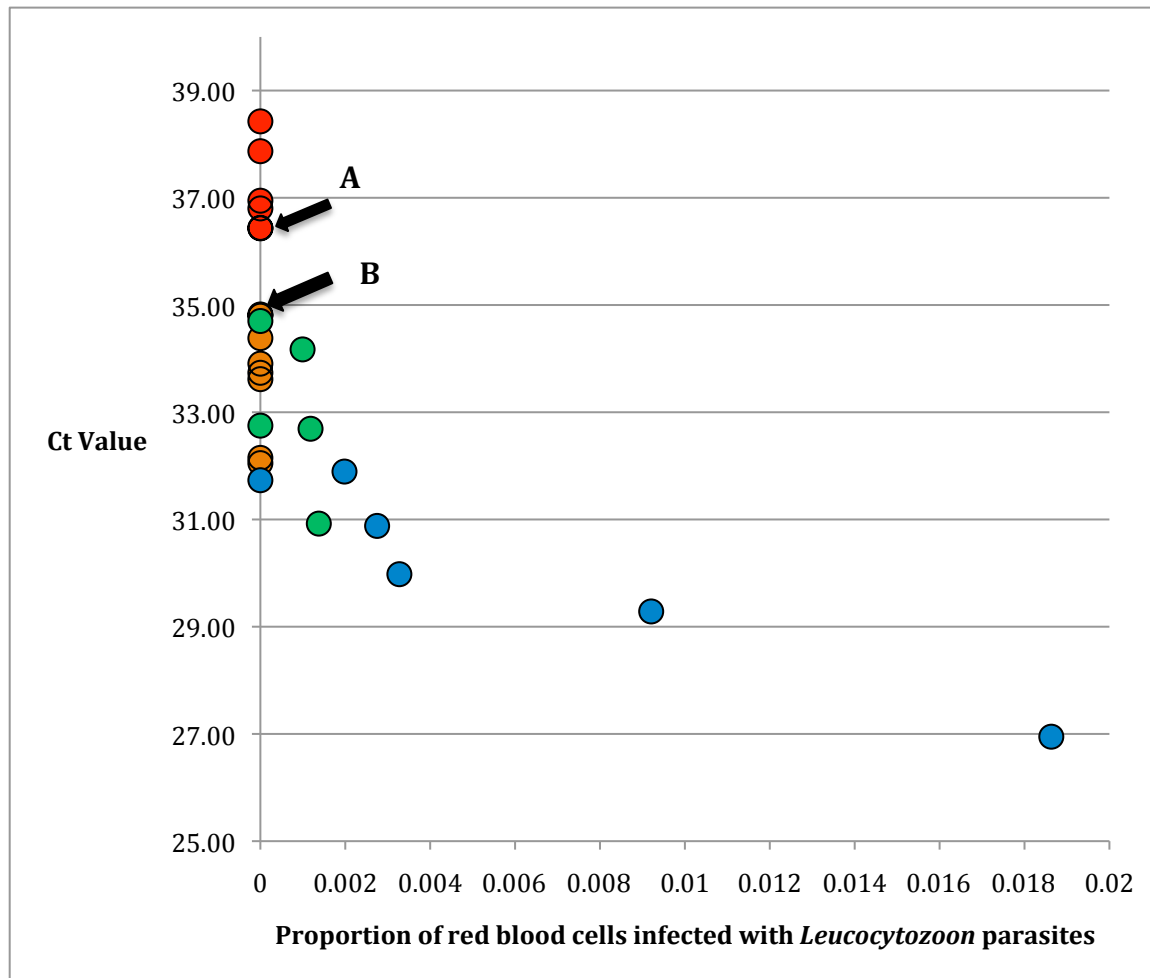


Figure 1: The relationship between Ct value and two different visual efforts to detect blood parasites on slides. A broad scan of a large area of the slide to detect any blood parasites wherein data are displayed as red circles (no parasites found by either observer and negative by nested PCR), orange circles (no parasites found by either observer and positive by nested), green circles (parasites found by one of the two observers and positive by nested PCR), or blue circles (parasites found by both observers and positive by nested PCR). The x-axis portrays parasitemia from a 400X magnification view of 100 fields. The points indicated by arrows labeled 'A' or 'B' indicate locations that overlap one another and cannot be seen.

GENERAL CONCLUSION

I screened 11 species of South American waterfowl from Peru and Argentina for infection by *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* parasites. Through the molecular characterization of hematozoa mtDNA *cyt-b* sequences, I was able to produce some of the first descriptions of these parasites in South American waterfowl species. The discovery of identical parasite haplotypes infecting waterfowl in both North and South America provides good evidence for the intercontinental transmission of these parasites by migratory waterfowl (Chapter 1 results), and my phylogenetic analyses of these parasite lineages combined with those already previously described shows evidence for possible host specificity of *Haemoproteus* and *Leucocytozoon* blood parasites in waterfowl. Additionally, the successful design and validation of a real-time, quantitative PCR methodology described here (Chapter 2) will provide a useful tool for researchers investigating pathogenic effects of haemosporidian infection in wild waterfowl species. Results showed that my qPCR protocol was more sensitive than microscopy in determining relative parasitemia rates in hosts harboring low to moderate levels of blood parasite infection.

My study of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* blood parasite infections in South American waterfowl found a much lower apparent prevalence rate (3.1%) than other studies examining these infections in waterfowl species around the globe (Bennett et al., 1981; Cumming et al., 2012; Greiner et al., 1975; Ramey et al., 2012). While this may seem unusual, similar rates have been observed in other regions of the Neotropics (White et al., 1978), and multiple environmental variables can have an effect on

blood parasite prevalence in avian species. Sampling methods, season, and sampling elevation are only some of the factors that can affect blood parasite prevalence. Perhaps the most interesting finding of this study is the presence of *Leucocytozoon* infections in South American waterfowl. As described in Chapter 1, the only species of *Leucocytozoon* known to infect anseriform hosts is *L. simondi*, which has previously been thought to be endemic to Holarctic regions (Valkiūnas, 2005). The fact that these *Leucocytozoon* lineages infecting South American waterfowl were identical to lineages previously identified in Tundra Swans (*Cygnus columbianus*) and Northern Pintails (*Anas acuta*) in North America, in tandem with results from examination of peripheral blood smears from *Leucocytozoon* infected hosts endemic to South America suggests that these observed infections are indeed *L. simondi*, which would provide strong evidence that *L. simondi* has a broader range than previously thought or has undergone some measure of range expansion and found suitable vectors to be transmitted from migratory North American hosts to South American waterfowl species.

The successful development and optimization of a qPCR protocol to estimate relative parasitemia rates of *Leucocytozoon* parasites in waterfowl blood could provide a tremendously useful tool for researchers examining pathogenic or sub-lethal effects of *Leucocytozoon* infection. While microscopy remains an essential tool for haemosporidian research, this new methodology will enable researchers who are not trained in microscopic techniques and haemosporidian taxonomy and identification to determine a host's relative parasitemia level quickly and relatively easily. By reporting my assay results as raw Ct values as opposed to transforming these data to more abstract estimates (e.g. Friedl and

Groscurth, 2011; Knowles et al., 2010b; Zehtindjiev et al., 2008), I suggest that other researchers can easily apply my techniques to their own research.

My results provide good evidence that qPCR can be more sensitive than microscopy at diagnosing and quantifying infection levels at low parasitemia (Chapter 2). Because we have very few samples that contained moderate levels of infection and no samples that could be considered heavily infected, it is unclear how the assay would perform on samples that are heavily parasitized. However, Figure 1 (Chapter 2) suggests that the method will continue to have potential at higher rates. The possibility to expand this methodology to include species of *Haemoproteus* and *Plasmodium* would be extremely beneficial, particularly through the use of multiplex PCR techniques which are possible using TaqMan primer/probe sets. This would benefit studies where only parasitemia data was of interest and molecular characterization of parasite haplotypes through DNA sequencing wasn't necessary. Multiplex reactions, which allow the simultaneous quantification of two or more fluorogenic probes would greatly reduce the amount of laboratory preparation needed to obtain data on multiple parasite genera.

This collection of work on avian haemosporidians greatly expands our limited knowledge of these infections in waterfowl. The data produced on hematozoa infections in South American waterfowl (Chapter 1) provide a good baseline for future studies, particularly those pertaining to intercontinental transmission of parasitic infections, as well as possible range expansion of *Leucocytozoon* parasites. The development and validation of my qPCR assay (Chapter 2) provides a new molecular tool for studies related to pathological or ecological effects of varying levels of hematozoa infections in waterfowl

and may, without a large amount of effort, produce to gather data on additional genera of parasites from a much broader range of avian hosts.

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